FORM PTO-1390
(REV 12-29-99)

U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

HSS-021XX

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 36 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/869638

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/10833	03 November 2000	04 November 1999

TITLE OF INVENTION

METHOD FOR THE AUTOMATIC ANALYSIS OF MICROSCOPE IMAGES

APPLICANT(S) FOR DO/EO/US

TIM WILHELM NATTKEMPER; HELGE RITTER; WALTER SCHUBERT

Applicant herewith submits to the United states Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))

   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).

   b. ☒ has been transmitted by the International Bureau.

   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))

   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).

   b. ☐ have been transmitted by the International Bureau.

   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.

   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.

   ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☐ Other items or information:

Express Mail Number

EL 751779567 US

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/869638	PCT/EP00/10833	HSS-021XX

17. [X]   The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

		CALCULATIONS	PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1,000.00**			
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**			
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**			
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**			
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**			

	CALCULATIONS	PTO USE ONLY
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 0	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	15 - 20 =	0	X $18.00	$ 0	
Independent claims	1 - 3 =	0	X $80.00	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+$270.00	$ 0	

	CALCULATIONS	PTO USE ONLY
TOTAL OF ABOVE CALCULATIONS =	$ 860.00	
[X] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.	$ 430.00	
SUBTOTAL =	$ 430.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	$ 0	
TOTAL NATIONAL FEE =	**$ 430.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property	$	
TOTAL FEES ENCLOSED =	$ 430.00	

	Amount to be	$
	Refunded:	
	Charged:	$

a. [X] A check in the amount of $ 430.00 _____ to cover the above fees is enclosed. A check in the amount of $_____ is enclosed for the assignment recordation fee.

b. ☐   Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X]   The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0804 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Customer Number 207

SEND ALL CORRESPONDENCE TO:

Weingarten, Schurgin, Gagnebin & Hayes LLP
Ten Post Office Square
Boston, Massachusetts 02109

Date: 7-27

SIGNATURE

Charles L. Gagnebin III
NAME

25,467
REGISTRATION NUMBER

Form PTO-1390 (REV 11-09) page 2 of 2   CLG/mc/253327-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application	:	TIM WILHELM NATTKEMPER, ET AL.
Application No.	:	
Filed	:	Herewith
For	:	METHOD FOR THE AUTOMATIC ANALYSIS
		OF MICROSCOPE IMAGES
Examiner	:	
Attorney's Docket	:	HSS-021XX

Group Art Unit:

* *
I hereby certify that this correspondence is being deposited
with the United States Postal Service as first class mail in an
envelope addressed to: Commissioner for Patents, Washington,
D.C. 20231 on _____.

By:_____
Charles L. Gagnebin III
Registration No. 25,467
Attorney for Applicant(s)

* *

PRELIMINARY AMENDMENT

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

Sir:

     Kindly enter the following Preliminary Amendment in the

above-identified application:

Express Mail Number
EL 751779567 US

In the Claims:

Please amend the Claims to read as follows (a copy of the amended claims showing the additions and deletions appears at the end for the Examiner's convenience):

3.   The method as claimed in claim 1 wherein the biological objects to be determined are marked with one or plural chemical markers before the microscope images are taken.

5.   The method as claimed in claim 3 wherein said chemical markers are fluorochrome markers and the microscope images are fluorescence images.

Please add the following new claims 14 and 15:

14.   The method as claimed in claim 2 wherein the biological objects to be determined are marked with one or plural chemical markers before the microscope images are taken.

15.   The method as claimed in claim 4 wherein said chemical markers are fluorochrome markers and the microscope images are fluorescence images.

REMARKS

This Preliminary Amendment puts the claims into proper form for examination.   Note that claims 3 and 5 have been amended; new claims 14 and 15 have been added; and claims 1, 2, 4, and 6-13 remain unchanged.   Kindly calculate the filing fee based on the amended claims.

The Examiner is encouraged to telephone the undersigned attorney to discuss any matter which would expedite allowance of the present application.

Respectfully submitted,

TIM WILHELM NATTKEMPER,
ET AL.
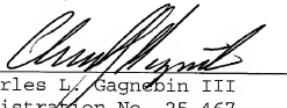
By: _____
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Date: 7-2-1

CLG/mc/253308-1
Enclosure

Red-lined Claims for the Examiner's convenience:

3.   The method as claimed in claims 1 ~~or 2~~ wherein the
biological objects to be determined are marked with one or
plural chemical markers before the microscope images are taken.

5. The method as claimed in claims 3 ~~or 4~~ wherein said chemical
markers are fluorochrome markers and the microscope images are
fluorescence images.

Nattkemper, T., et al.
U.S. Patent Application

5   METHOD FOR THE AUTOMATIC ANALYSIS OF MICROSCOPE IMAGES

10   The invention relates to a method for the automatic
analysis of microscope images of biological objects, in
particular for the analysis of fluorescence images of
cells.

          In numerous experiments in the field of modern
biomedical research, fluorescence techniques, amongst
15   others, are used for marking in order to make
biologically significant objects visible under the
microscope. Such objects may be cells of a certain type
or of a certain state, for example. In recent years,
the progress made in automating such experiments has
20   made it possible for biomedical laboratories to perform
large numbers of such image-producing experiments fully
automatically.

          DE 197 09 348 C1, for example, describes a
fluorescence microscope technique which produces a set
25   of n images of a single sample by preparing said
lymphocyte sample with n different fluorochrome markers.
In each image, different lymphocyte subsets will be
fluorescent and appear to have shining outlines. Each
lymphocyte in the sample will have its specific
30   fluorescent behavior in the image set. It will appear
fluorescent in one subset of images, otherwise remaining
invisible in the other images of the set.

For extracting the fluorescence patterns from the image set, the fluorescent lymphocytes will first of all have to be detected in the n images. The fluorochrome marked lymphocytes differ in number, location and intensity. Since such vast numbers of images and image data will result, from which the information will first of all have to be extracted for biological interpretation later on, there will be a so-called bottleneck in the evaluation of the experiments. Image interpretation by human employees is impractical since it is too time-consuming and the results are often not reliable. This is due to the visual evaluation work which is tiring and will lead to concentration losses already after a short time only. Moreover, the objects to be detected differ in number, location and intensity. Consequently, image parameters such as contrast and noise will differ from image to image. Furthermore, the objects, for example cells in tissue samples, vary considerably as regards their shape and size.

Consequently, there is a need for automatic evaluation methods which are capable of locating the objects to be detected in one image.

Earlier work on the automation of cell detection essentially focused on model-based approaches. Numbering amongst these is also the idea to adapt a geometric model to a gradient ensemble (Mardia et al., 1997, In: IEEE Transactions of Pattern Analysis and Machine Intelligence, 19: 1035-1042). This also includes the exploitation of wave propagation (Hanahara and Hiyane, 1990, In: Machine Visions and Applications, 3: 97-111) or a Hough transformation for detecting circular objects (Gerig and Klein, 1986, In: Proc. Int.

Conf. on Pattern Recognition, 8: 498-500). These approaches, however, have the disadvantage that they are frequently susceptible to changes in the shape of the object, and they may not be readily adapted by persons not skilled in the art. Furthermore, the images will not infrequently contain noise, owing to heterogeneous lighting conditions, and the cells will be partly obscure, which makes detection by boundary scanning unsuitable (Galbraith et al.; 1991, In: Cytometry, 12: 579-596).

It is therefore the object of the present invention to provide a method of the aforementioned kind comprising a simple automatic cell detection procedure which is fast and readily adapted.

This object is accomplished by a method having the features set out in claim 1.

Advantageous embodiments are described in the sub-claims.

The present invention relates to a method for the automatic analysis of microscope images of biological objects, in particular for the analysis of fluorescence images of cells, comprising the following steps: a) at least two microscope images are taken of a sample containing a plurality of biological objects; b) a first microscope image is selected and the positions of mass gravity centers of a number n of the individual objects visible in said first microscope image are marked, in which process each marked object is assigned a defined first image excerpt completely surrounding the marked object, and in which each first image excerpt containing a marked object is assigned the value 1, with the number n of such marked first image excerpts constituting a

positive training set; c) a number m of second image excerpts which are spaced a predetermined minimum distance from the first image excerpts are selected and marked, with the size and shape of a second image excerpt corresponding to the size and shape of a first image excerpt, in which process each second image excerpt is assigned the value 0, with the number m of such marked second image excerpts constituting a negative training set; d) characteristic features and/or feature combinations of the positive and negative training sets are determined and assigned to a classification value between 0 and 1, said classification value representing the degree of probability of the presence of a marked object, and the determined features and/or feature combinations are stored; e) classification values of all image points of the second and each further microscope image are automatically determined by comparing the image data of the second and each further microscope image with the features and/or feature combinations determined in procedural step d), in which process the classification value for an image excerpt surrounding the image point is determined for each image point of the second and each further microscope image, said image excerpt corresponding in size and shape to said first or second image excerpt; and f) the position(s) of biological objects in the second or each further microscope image is/are recognized by evaluating classification values, said determined classification values being then compared with a predetermined threshold value which represents the presence of a biological object.

The method of the invention for detecting biological objects such as cells in microscope images advantageously uses classification values which will assign to image excerpts of given size and shape a value
5   between 0 and 1 and which will indicate whether (1) or not (0) a biological object can be seen in this image excerpt. However, it is also possible to use any other numerical values which are different from each other. In an image, all cells are found by assigning all image
10   areas to one classification value. A simple automatic search for high values (threshold value analysis) will thus allow the positions of the biological objects to be located automatically and stored in a file and processed further, for example. Providing such classification
15   values, or a similar classifier, and simply adapting them in case of significant changes in the microscope images due to microscope modifications, different samples, different fluorescence markers or different cell types is made possible by the use of the method of
20   the invention which constitutes an artificial neural network.

The method of the invention will allow a fully automatic detection of biological objects such as cells in microscope images, after it has been trained by a
25   user by being provided with a set of cell-positive image excerpts. It is particularly easy to use since it merely requires the user to mark cells on the screen for training, which is a routine activity in laboratory work. The system is thus suitable for the fast
30   evaluation of numerous microscope images for localizing cells. Owing to its ease of use, the method of the invention can be readily applied and executed. It is

suitable for supporting any kind of empirical work with microscope images of biological objects at universities as well as in industry – which sectors have a vast throughput of samples and require objective and reproducible evaluations.

In a further advantageous embodiment of the method of the invention, the sample is a tissue sample and the biological object is a cell. In particular for automatic cell detection, the method of the invention has shown to be advantageous over conventional methods of the prior art.

In yet another advantageous embodiment of the method of the invention, the biological objects to be determined are marked with one or plural chemical markers before the microscope images are taken. In this process, a bleaching or rinsing operation may be performed between the taking of the individual microscope images. The chemical markers may be fluorochrome markers and the microscope images may be fluorescence images.

For example, fluorochrome markers are used on lymphocytes in order to determine the presence of proteins in lymphocyte cell surfaces. Each marker will bind to a lymphocyte subset which depends on the existence of the respective protein in the lymphocyte surface membrane. Fluorescence excitation will cause the binding lymphocytes to appear with high intensities. A corresponding microscope image will be taken by a CCD camera. The marker will subsequently be removed from the cells by means of bleach, and the operation will be repeated using a different marker. In the embodiment described here the process comprising marking, image

generation and bleaching can be repeated with up to nine
markers. During each repetition, the lymphocyte
positions will remain unchanged, which allows a matching
or coincidence comparison of the positions in the
different images. This experimental setup is especially
used for analyzing T lymphocytes which have invaded the
muscular tissue in a clinical case of sarcoidosis. The
lymphocytes were prepared with n=7 markers, and seven
fluorescence images were taken, in which different
subsets of the cells appeared fluorescent. On
conclusion of all n steps, a set of n microscope images
with different lymphocyte subsets can be evaluated. The
n microscope images are evaluated by means of the method
of the invention.

In this case, the number n of the individual
biological objects, in particular cells, marked in
procedural step b) will be larger than or equal to 50.
For each marked point, it will be decided whether or not
such point constitutes a cell center. Since the
lymphocytes, for example, will be in organic fiber
material during an invasion situation, they will not be
circular in shape, but irregularly oblong, which shape
is referred to as predominantly convex. For the
classification of the biological objects, a special form
of a neural network, the so-called "Local Linear Map"
(LLM) (Ritter, 1991: In: Artificial Neural Networks,
Elsevier Science Publishers B.V.) is used. The LLM
classifier is trained by a set of image excerpts which
contain cells. For training the LLM classifier,
according to procedural step b) an image is selected
from the image set. Furthermore, e.g. by means of a
computer mouse, a group of fluorescing cells are marked.

The group of image excerpts dimensioned N x N surrounding these cells will constitute the set of positive training examples or a positive training set. The first image excerpt is square in shape, according to an advantageous embodiment of the method of the invention, with the size N x N or the side length N of the first image excerpt corresponding to the maximum diameter of the biological objects in the first microscope image.

A further set of second image excerpts is randomly selected fully automatically from the same image, according to procedural step c), thereby keeping a minimum distance of e.g. 3 - 5 pixels from the already marked first image excerpts. This second set constitutes the set of negative training examples or a negative training set. According to an advantageous embodiment of the invention, the number m of second image excerpts will be larger than or equal to 50, with the second image excerpts being defined automatically, at the same time keeping to the minimum distance from the respective first image excerpts.

In an embodiment, a d_{in} dimensional feature vector x will be calculated for each training example excerpt. The set of selected image excerpts will be subjected to a principal component analysis (PCA). This is a known technique for classification tasks in computer vision. The basic idea of PCA is that the high-dimensional image excerpt is mapped to a considerably smaller dimensional (d_{in} = approx. 6) feature space. These features will give so-called input feature vectors x. The feature vectors have clearly less data than the actual image excerpts. Calculation of the feature vectors for the

positive and negative input examples will result in the training set of the (input, output) pair

$$\Gamma = \{(x_\alpha, y_\alpha)\}_\alpha.$$

For the positive training set, y_α will be set to equal 1, and to equal 0 for the negative ones.

The LLM is defined by

$$\{w_i^{in} \in R^d_{in}, w_i^{out} \in R^d_{out}; A_i \in R^{d_{in} \times d_{out}}, i = 1...l\}.$$

A triple $v_i = (w_i^{in}, w_i^{out}, A_i)$ is referred to as a node. For training the LLM, a pair (x_α, y_α) is randomly selected from Γ, and the learning rules

$$\Delta w_k^{in} = \epsilon^{in}(x_\alpha - w_k^{in}) \quad\quad (1)$$

$$\Delta w_k^{out} = \epsilon^{out}(y_\alpha - y(x_\alpha)) + A_k \Delta w_k^{in} \quad\quad (2)$$

$$\Delta A_k = \epsilon^A (y_\alpha - y(x_\alpha)) \; \frac{(x_\alpha - w_k^{in})^T}{||x_\alpha - w_k^{in}||^2} \quad\quad (3)$$

will be implemented. ϵ^{in}, ϵ^{out}, $\epsilon^A \subset]0,1[$ are descending learning step sizes and k is true for $k = \arg\min_k \{||x - w_k^{in}||\}$. Therefore, w_k^{in} is the nearest neighbor to input x. This is repeated $l*10,000$ times.

The trained LLM classifier performs a mapping of fluorescence image points to classification or evidence values in $(0;1)$. For calculating the classification value for e.g. a fluorescent cell at an image point, the feature vector x for its surrounding area will be calculated. The LLM output for the input x is calculated by

$$y(x) = w_k^{out} \ x \ A_k(x - w_k^{in}) \qquad (4)$$
$$\text{with } k = \arg \min_k\{||x - w_k^{in}||\}$$

In the embodiment described, the number of nodes is $i = 5$, and the image excerpt size is N=15 pixels.

For the detection of all biological objects, e.g. fluorescent cells, in the second or each further image of the image set, each image point is mapped to its classification value by equation (4). The surrounding image region of the point will be supplied to the classifier which will then calculate its classification value. By calculating the classification values for each point in an image, a so-called classification or evidence map of the image will be obtained. Regions of high classification values or evidences will indicate biological objects such as fluorescent cells in the fluorescence image in question. All points of a classification value higher than e.g. 0.5 and without major evidences in their vicinity will form the set of positions of biological objects such as fluorescent cells in the image. A similar approach will be adopted for all further microscope images of the image set.

In accordance with an advantageous embodiment of the invention, the classification values of all image

points of the second and each further microscope image are determined automatically according to procedural step e) by scanning the image surface of the second and each further microscope image.

In another advantageous embodiment of the method of the invention, the object positions determined by the procedural steps a) to f) are compared in the totality of the microscope images – which will determine the spatial position and the distribution of the individual objects in the sample.

This will allow fluorescence patterns of cells, for example, to be determined. In doing so, after the cell detection process in all images, locally corresponding fluorescence sites are found in different images so as to map fluorescences of the same cell to its marker combination pattern. This coincidence analysis is exclusively based on the positions of the cells in the images, and is a difficult task since the determined positions of one and the same cell will not always be exactly identical in different images. Furthermore, the number of fluorescent cells in the images will vary strongly. Both aspects make a simple matching on the basis of exclusively the detected cell positions impossible. The method of the invention avoids such problems by evaluating the classification values or evidences of all images of the image set.

By determining the classification values or evidences of all n images as described above, one will obtain n classification or evidence values for each point. Subsequently, the maximum evidence value for each point will be selected and entered into a new evidence map at its coordinates. This map is referred

to as "Master Evidence Map" since each biological object, for example a cell, which occurred in at least one of the images, will be represented by a high evidence value in this map. Applying procedural step f)

5   to said "Master Evidence Map" will result in all positions of the biological objects. This set of M cell positions $\{(x_i, y_i)\}$ is referred to as the "master set". Subsequently, the binary fluorescence values $f_j^{(i)}$ will be collected for each biological object from the "master

10   set". f_j of $p_i = (f_1^{(i)}, \ldots, f_n^{(i)})$ will be set to 1 when a biological object such as a cell in the j-th fluorescence image of the image set was detected in close vicinity to its coordinates (x_i, y_i). A simple local matching procedure between the master set and the

15   classification values of the positions of the biological objects, or cell positions, detected for all fluorescence images will give the binary fluorescence patterns of all detected objects or cells.

       One use, according to the invention, of the

20   described new method for the automatic analysis of microscope images of biological objects is the automatic cell classification of fluorescent cells. For example, a set of seven fluorescence images was taken according to the method of the invention. The seven different

25   markers are cd2, cd3, cd4, cd8, cd11b, cd19 and cd26; these are antibody markers commonly used in fluorescence microscopy. The training set of all cell excerpts was selected by hand, from the cd4 image, to be precise. In each image, the fluorescent cells were detected by means

30   of the LLM classifier. Using the maximum condition of the classification values or evidences calculated by the LLM, the "Master Evidence Map" was generated. The

positions of M = 550 fluorescent cells were then extracted from said "Master Evidence Map". Finally, the local matching step produced the marker combination pattern p_j, $j = 1, ..., 550$.

For examining the distribution of the marker combination patterns within the lymphocyte group, their binary patterns $(f_1^{(i)}, ..., f_7^{(i)})$ were mapped from the dual system to a numerical label in the decimal system. The pattern frequencies were counted and illustrated in the form of a histogram (histogram including $2^7 = 128$ bars). This has shown that only 24 of 128 possible patterns in the total lymphocyte group were found. Three patterns dominate in number, (1000000) (= 1), (0010000) (= 8) and (1010000) (= 9). These are cells which bound to only cd2 or cd4, or to both these markers only. The remaining frequencies are below 30.

In order to get an idea of the coincidence of a certain marker compared to the remaining markers, a corresponding histogram was calculated for each marker. The absolute number of cells which were made fluorescent by this selected marker were shown. It was possible to determine that the absolute number of fluorescent cells will vary strongly. Most dominant are the markers cd2 and cd4. Lymphocytes fluorescence-marked with cd19 occur seldom and only once coincide with another marker (cd2). This means that this marker is highly selective. A strong coincidence may also be observed for example between the pairs (cd2, cd8), (cd3, cd8) and (cd2, cd3).

In the embodiment described, microscope images of a single visual field were recorded and analyzed. However, if one is confronted with an enormous number of several hundred visual images, for example, then the

method of the invention will allow a precise statistical analysis of those immune cell subsets which have penetrated the tissue sites, within a relatively short time.

What is claimed is:

1. A method for the automatic analysis of microscope images of biological objects, in particular for the analysis of fluorescence images of cells, comprising the following steps:

   a) taking at least two microscope images of a sample including a plurality of biological objects;

   b) selecting a first microscope image and marking the position(s) of mass gravity centers of a number n of the individual objects discernible in the first microscope image, in which step each marked object is assigned a defined first image excerpt which completely surrounds the marked object, and each first image excerpt including a marked object is assigned the value 1, with the number n of such marked first image excerpts constituting a positive training set;

   c) selecting and marking a number m of second image excerpts each spaced a predetermined minimum distance from said first image excerpts, with a second image excerpt corresponding in size and shape to said first image excerpt, in which step each second image excerpt is assigned the value 0, with the number m of such marked second image excerpts constituting a negative training set;

   d) determine characteristic features and/or feature combinations of the positive and negative training sets and assigning said characteristic features and/or feature combinations to a classification value between 0 and 1, said classification value representing the degree of probability of the

presence of a marked object, and the determined features and/or feature combinations are stored;

e) determine classification values of all image points of the second and each further microscope image by comparing the image data of the second and each further microscope image with the features and/or feature combinations determined in procedural step d), in which step, for each image point of the second and each further microscope image, the classification value for an image excerpt surrounding the image point is determined and the size and shape of this image excerpt corresponds to the size and shape of the first or second image excerpt; and

f) recognizing the position(s) of biological objects in the second or each further microscope image by evaluating the determined classification values, in which step the determined classification values are compared with a given threshold value representing the presence of a biological object.

2. The method as claimed in claim 1 wherein the sample is a tissue sample and the biological object is a cell.

3. The method as claimed in claims 1 or 2 wherein the biological objects to be determined are marked with one or plural chemical markers before the microscope images are taken.

4. The method as claimed in claim 3 wherein the objects to be determined are marked with one or plural chemical markers before the microscope images are taken, with a

bleaching or rinsing procedure being performed between the taking of the individual microscope images.

5. The method as claimed in claims 3 or 4 wherein said chemical markers are fluorochrome markers and the microscope images are fluorescence images.

6. The method as claimed in claim 1 wherein the microscope images are taken by a CCD camera and then digitized.

7. The method as claimed in claim 1 wherein the number n of the individual biological objects marked in procedural step b) is larger than or equal to 50.

8. The method as claimed in claim 1 wherein the first image excerpt is of square shape, with the size and/or side length of the first image excerpt corresponding at least to the maximum diameter of the biological objects in the first microscope image.

9. The method as claimed in claim 1 wherein the number n of second image excerpts is larger than or equal to 50, with the second image excerpts being defined automatically, keeping to the minimum distance from the respective first image excerpts.

10. The method as claimed in claim 1 wherein classification values of all image points of the second and each further microscope image are automatically

determined according to procedural step e) by scanning the image surface of the second and each further microscope image.

11. The method as claimed in claim 1 wherein the threshold value of the classification value representing the presence of a biological object is at least 0.5.

12. The method as claimed in claim 1 wherein the object positions determined by procedural steps a) to f) are compared in the total number of microscope images so as to obtain a spatial location and distribution of the individual objects in the sample.

13. Use of a method as claimed in claim 1 for the automatic cell classification of fluorescent cells.

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                                                   Attorney
                                                   Docket No.: HSS-021XX

<u>DECLARATION AND POWER OF ATTORNEY</u>

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed
below) or an original, first and joint inventor (if plural names are listed below) of
the subject matter which is claimed and for which a patent is sought on the invention
entitled: **METHOD FOR THE AUTOMATIC ANALYSIS OF MICROSCOPE IMAGES**

the specification of which (check one):

[ ] is attached hereto.   [X] was filed 7/2/01   as Application No. 09/869,638
                              amended on _____ (if applicable).

[X] was filed as PCT International Application No. PCT/EP00/10833 on 03 November 2000,
    and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-
identified specification, including the claims, as amended by any amendment referred
to above.

I acknowledge the duty to disclose information which is material to the patentability
of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, USC §119(a)-(d) of any
foreign application(s) for patent or inventor's certificate listed below and have also
identified below any foreign application for patent or inventor's certificate having a
filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)   Date Filed   Priority Claimed

 199 53 181.1 Germany   04 November 1999   [X]   [ ]
(Number)   (Country)   (Day/Month/Year)   Yes   No

_____   _____   [ ]   [ ]
(Number)   (Country)   (Day/Month/Year)   Yes   No

I hereby claim the benefit under Title 35, USC §119(e) of any United States
provisional application(s) listed below:

_____   _____
(Application Number)   (Filing Date)

_____   _____
(Application Number)   (Filing Date)

_____   _____
(Application Number)   (Filing Date)

_____   _____
(Application Number)   (Filing Date)

                                        Express Mail Number

                                        EV 009948517 US

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                                                   Attorney
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I hereby claim the benefit under Title 35 USC §120 of any United States application(s)
listed below and insofar as the subject matter of each of the claims of this
application is not disclosed in the prior United States application in the manner
provided by the first paragraph of Title 35 USC §112, I acknowledge the duty to
disclose material information as defined in Title 37, Code of Federal Regulations,
§1.56(a) which occurred between the filing date of the prior application and the
national or PCT international filing date of this application:

(Application No.)	(Filing Date)	(Patented/pending/abandoned)
(Application No.)	(Filing Date)	(Patented/pending/abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) to
prosecute this application and transact all business connected therewith in the Patent
and Trademark Office, and to file with the USRO any International Application based
thereon.

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I hereby declare that all statements made herein of my own knowledge are true and that
all statements made on information and belief are believed to be true; and further
that these statements were made with the knowledge that willful false statements and
the like so made are punishable by fine or imprisonment, or both, under Section 1001
of Title 18 of the United States Code and that such willful false statements may
jeopardize the validity of the application or any patent issued thereon.

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                                                   Docket No.:   HSS-021XX

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CLG/mc/253299-1

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Attorney
Docket No.:   HSS-021XX

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